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Award Number: W81XWH-04-1-0705

TITLE: A Novel Membrane-Permeable, Breast-Targeting, Pro-Apoptotic Peptide for Treatment of Breast Cancer

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REPORT DATE: October 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
10-29-2007	Final	Sept 30,2004 – Sept 29,2006

4. TITLE AND SUBTITLE

A Novel Membrane-Permeable, Breast-Targeting, Pro-Apoptotic Peptide for Treatment of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0705

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

North Dakota State University
Fargo, ND 58105

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this project, we tried to create a novel peptide with three properties: membrane permeable, breast targeting, and inducing apoptosis. The peptide is expected to be able to induce apoptosis specifically in breast cancer cells and will be tested as a single therapeutic agent as well as in combination with chemotherapeutic drugs to treat breast cancer. For specific aim 1, we have synthesized the breast-targeting, membrane permeable, pro-apoptotic peptide. The BH3 peptide of Bid (EDIIRNIARHLAQVGDSMDR) has been synthesized with eight d-arginine residues at the N-terminus with a glycine linker residue, followed by a breast-homing sequence (CPGPEGAGC) at the C-terminal. A control peptide with a mutation in the BH3 domain was also synthesized, r8-BH3(L/E)-CPGPEGAGC. Next, we have tested the therapeutic efficacy of the peptide in treatment of breast cancer. The peptide was tested for apoptosis induction first in vitro in cultured breast cancer MCF-7 cells. However, this peptide failed to induce apoptosis in MCF-7 cells at the concentrations being tested. We tried to produce an alternative peptide, which has similar design and predicted functions but with stronger apoptosis inducing capability. The synthesis of the new peptide was not completed. In parallel studies, we have developed a novel strategy using Valproic acid to enhance apoptosis induced by TRAIL in cancer cells. The effects of VPA on apoptosis appear to be due to the down-regulation of survivin and Bcl-XL

15. SUBJECT TERMS
Breast cancer, apoptosis, BH3 peptide, breast targeting

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**		11	USAMRMC
					19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The Bcl-2 family proteins are key regulators of apoptosis. Bid, a pro-apoptotic member of the Bcl-2 family, induces apoptosis through its Bcl-2 homology 3 (BH3) domain. The Bid BH3 peptide is able to induce apoptosis in cancer cells when it is linked to a membrane permeable peptide. A breast-homing peptide, CPGPEGAGC, is shown to specifically target breast tissue. This project aims to design and test a novel therapeutic peptide for breast cancer, which with three properties: membrane permeable, breast targeting, and inducing apoptosis.

Final report

For specific aim 1, we have synthesized the breast-targeting, membrane permeable, pro-apoptotic peptide. The BH3 peptide of Bid (EDIIRNIARHLAQVGDSMDR) has been synthesized with eight arginine residues at the N-terminus linked by a glycine linker residue, followed by a breast-homing sequence (CPGPEGAGC) at the C-terminal. A control peptide with a mutation in the BH3 domain was also synthesized, r8-BH3(L/E)-CPGPEGAGC. Next, we have tested the therapeutic efficacy of the peptide in treatment of breast cancer. The peptide was tested for apoptosis induction in cultured breast cancer MCF-7 cells. Cells were treated with various concentrations of peptide ranging from 1 μM to 100 μM for 24 hours. Unfortunately, this peptide failed to induce apoptosis in MCF-7 cells at these tested concentrations (Figure 1). The peptide was also tested in T47D and ZR-75-1 breast cancer cells, in which they too failed to induce apoptosis. It's not practical to increase the concentration above 100 μM, since higher concentration will not be achieved in vivo in mice or in clinic. We have examined whether the peptide has entered the cells by western blotting using a polyclonal antibody raised against Bid BH3 domain (Abgent, San Diego, CA). As shown in Figure 2, the peptide has entered the cells successfully. The results indicate that breast-homing sequence (CPGPEGAGC) may interfere with the pro-apoptotic activity of the BH3 peptide.

To test whether the peptide can bind to Bax, we performed a co-immunoprecipitation experiment. As shown in Figure 3, the BH3 peptide is able to bind to Bax. The data suggest that the breast-homing sequence (CPGPEGAGC) may interfere with the activation of Bax by the BH3 domain, rather than the interaction with Bax.

It is also possible that the pro-apoptotic activity of the BH3 peptide is too weak to induce apoptosis. We have planned to synthesize an alternative peptide, which has similar design and predicted functions but with stronger apoptosis inducing capability. The new peptide has a hydrocarbon-stapled BH3 helix, produced by ruthenium-catalyzed olefin metathesis (1). This "stapled" BH3 peptide has been shown to have enhanced apoptosis-inducing activity and also is cell permeable. Thus, the eight arginine residues are no longer needed. The breast-homing sequence (CPGPEGAGC) will be added at the C-terminal. However, the synthesis of this peptide was not completed due to cost and changes happened to our collaborator.

During the time when we tested the designed peptide for inducing apoptosis in cancer cells, in a parallel study, we explored other strategies of enhancing apoptosis in tumors.

TNF-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent (2), which can selectively induce apoptosis in cancer cells while sparing normal cells. However, TRAIL is not always effective in inducing apoptosis in solid tumors. We have examined whether Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor (3), can enhance apoptosis induced by TRAIL in cancer cells.

Valproic Acid Enhanced TRAIL-induced Apoptosis. ZR-75-1 cells were resistant to TRAIL-induced apoptosis (Figure 4). Valproic acid (VPA) has been demonstrated to induce apoptosis in several types of cancers. We first examined the effect of treatment with VPA alone on ZR-75-1 cells. Treatment of ZR-75-1 cells with VPA did not induce cell death at concentrations of 0.5 – 6 mM for 24 h, but inhibited cell growth (Figure 4A). When ZR-75-1 cells were pre-treated with 2 mM VPA for 24 h and followed by exposure to various concentrations of TRAIL for additional 24h, significant level of apoptosis was induced (Figure 4B).

VPA Down-regulated Survivin and Bcl-X_L in ZR-75-1 Cells. To determine how VPA enhances TRAIL-induced apoptosis, we examined the effects of VPA on the expression of apoptosis regulators in ZR-75-1 cells. ZR-75-1 cells were treated with 2 mM VPA for 48 h. Expression of apoptosis regulatory proteins at various time points after VPA exposure was determined by western blotting (Figure 5). The levels of anti-apoptotic proteins survivin and Bcl-X_L were significantly decreased after VPA exposure, while the levels of other proteins, such as Bax and XIAP did not change. Expression of proteins of the death receptor family, DR5 and Fas, did not change after VPA exposure.

Survivin and Bcl-X_L Regulate TRAIL-induced Apoptosis. To determine whether survivin and Bcl-X_L are critical to VPA's effects on TRAIL-induced apoptosis, we knocked down these two proteins with small interference RNA. Treatment of ZR-75-1 cells with siRNAs specifically targeting survivin and Bcl-X_L successfully decreased the levels of the two proteins (Figure 6A and 6B). Treatments with survivin or Bcl-X_L siRNAs significantly enhanced TRAIL-induced apoptosis in ZR-75-1 cells (Figure 6A and 6B). Conversely, overexpression of survivin blocked apoptosis in ZR-75-1 cells treated with VPA plus TRAIL (Figure 6C). Overexpression of Bcl-X_L had little effects on TRAIL-induced apoptosis (Figure 6D).

VPA Down-regulated Survivin and Bcl-X_L by Increased Protein Degradation. To investigate the mechanism of VPA-induced down-regulation of survivin and Bcl-X_L, we examined the effects of VPA on the transcription of these two genes. ZR-75-1 cells were treated with 2mM VPA for 24h, and the levels of survivin and Bcl-X_L mRNA were determined by real-time PCR. VPA treatment did not suppress survivin or Bcl-X_L gene expression. Instead, the mRNA levels of both survivin and Bcl-X_L were increased after VPA exposure (Figure 7A and 7B). To determine whether proteasome-mediated protein degradation is involved in VPA-induced down-regulation of survivin Bcl-X_L, we treated

ZR-75-1 cells with VPA plus proteasome inhibitor MG-132. As shown in Figure 7C, MG-132 prevented VPA-induced down-regulation of survivin and Bcl-X_L.

Thus, we found a new strategy to use VPA to enhance TRAIL-induced apoptosis in cancer cells. This combined treatment may prove to be an effective and safe treatment of cancer.

The following people are personnel who have received pay from the research effort:
Graduate students: Binfeng Xia, Tianying Zhu.
Undergraduate students: Jyoti Iyer, Jenna Schmidt.
Post-doc associate: Xiangwei Huang.
PI: Bin Guo.

Key research accomplishments

The originally designed BH3 peptides have been synthesized and tested for in vitro apoptosis-inducing activity. The peptide was tested in three breast cancer cell lines, MCF-7, T47D, and ZR-75-1, but it failed to induce apoptosis in all these cells. A new peptide with improved activity has been designed, but synthesis of this peptide was not completed.

We have developed a novel strategy using Valproic acid to enhance apoptosis induced by TRAIL in cancer cells. Pre-treatment of ZR-75-1 cells with VPA significantly enhanced apoptosis induced by TRAIL. The increase of cell death was associated with a significant decrease in the protein levels of anti-apoptotic proteins survivin and Bcl-X_L as a result of pre-exposure to VPA. Down-regulation of survivin or Bcl-X_L by siRNAs sensitized ZR-75-1 cells to TRAIL-induced apoptosis. Moreover, overexpression of survivin blocked TRAIL-induced apoptosis. We further demonstrated that VPA-induced down-regulation of survivin and Bcl-X_L occurred not at the level of transcription but through proteasome-mediated protein degradation. Thus, VPA enhances TRAIL-induced apoptosis in ZR-75-1 cells by down-regulation of survivin and Bcl-X_L.

Reportable outcomes

The proposed peptide (RRRRRRRRG-EDIIRNIARHLAQVGDSMDR-CPGPEGAGC) and a control peptide RRRRRRRRG-BH3(L/E)-CPGPEGAGC were synthesized and tested in breast cancer cells.

Manuscript in preparation: Tianying Zhu et al., Valproic acid enhances TRAIL-induced apoptosis in ZR-75-1 cells.

Conclusions

We have synthesized the proposed peptides and tested the peptides in three breast cancer cell lines (MCF-7, T47D, and ZR-75-1) for induction of apoptosis. However, the peptide has no apoptosis-inducing activity in the current design, even though it can be delivered into cells. A new peptide with enhanced apoptosis-inducing capability was designed but its synthesis was not completed.

We have developed a novel strategy to use VPA to enhance TRAIL-induced apoptosis in cancer cells. The effects of VPA on apoptosis appear to be due to the down-regulation of survivin and Bcl-X_L.

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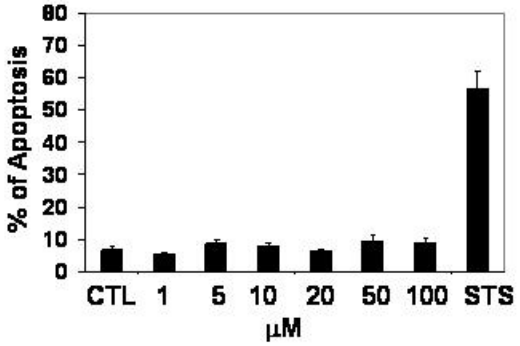

Figure 1. The peptide failed to induce apoptosis in MCF-7 cells. Cells were treated with various concentrations of the peptide for 24 hours. Apoptosis was examined by TUNEL assay. Treatment with 1 μM Staurosporin (STS) was used as a positive control for apoptosis.

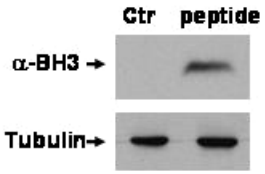

Figure 2. The peptide was able to enter the cells. MCF-7 cells were treated with 50 μM peptide for 24 hours. Cells were washed for five times with PBS and protein lysates were analyzed by western blot with a polyclonal antibody against Bid BH3 domain.

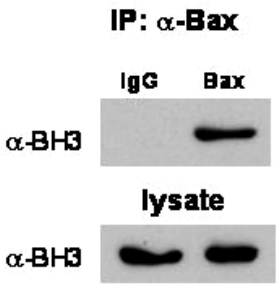

Figure 3. The peptide was able to bind Bax. MCF-7 cells were treated with 50 μM peptide for 24 hours. Co-immunoprecipitation was performed using anti-Bax antibody and the co-immunoprecipitates were analyzed by western blot with a polyclonal antibody against Bid BH3 domain.

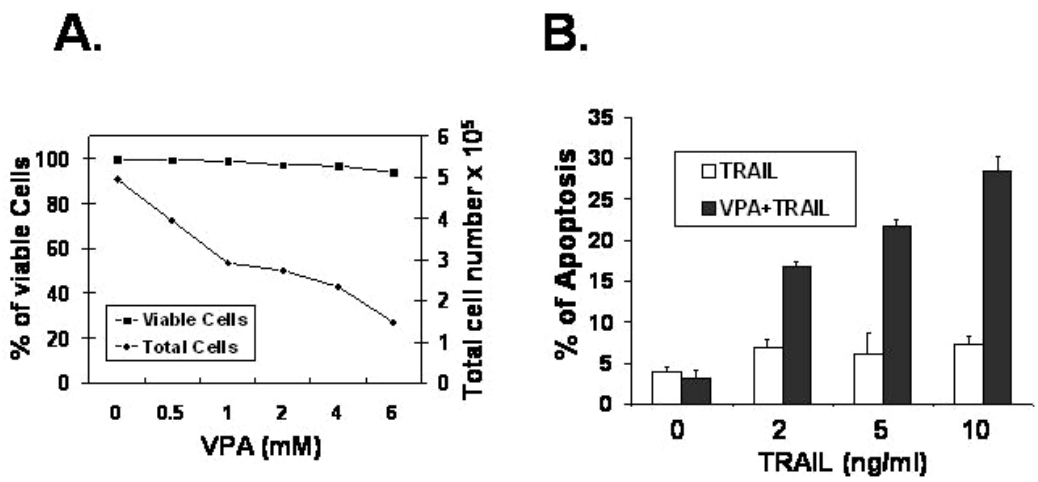

Figure 4. VPA enhances TRAIL-induced apoptosis in ZR-75-1 cells. **A,** ZR-75-1 cells were treated with different doses of VPA for 24 h. Cell viability and total cell number were assessed by a Guava PCA microcytometer using Guava® ViaCount reagent. **B,** ZR-75-1 cell were treated with PBS or 2mM VPA for 24 h, and then further treated with different amounts of TRAIL for additional 24 h. Apoptotic cells were counted by DAPI staining under a confocol microscope. Data represent mean value of three replicates.

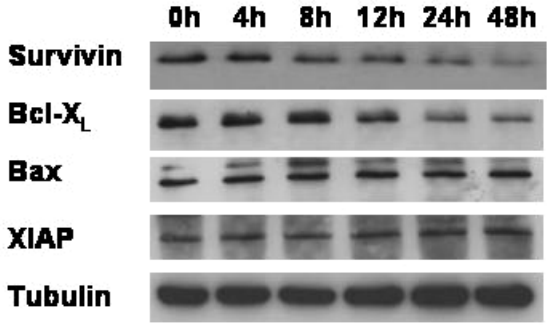

Figure 5. VPA down-regulates survivin and Bcl-X_L. ZR-75-1 cells were treated with 2mM VPA for 48 hours. At 4h, 8h, 12h, 24h, and 48 h after VPA exposure, cells were collected for western blotting analysis.

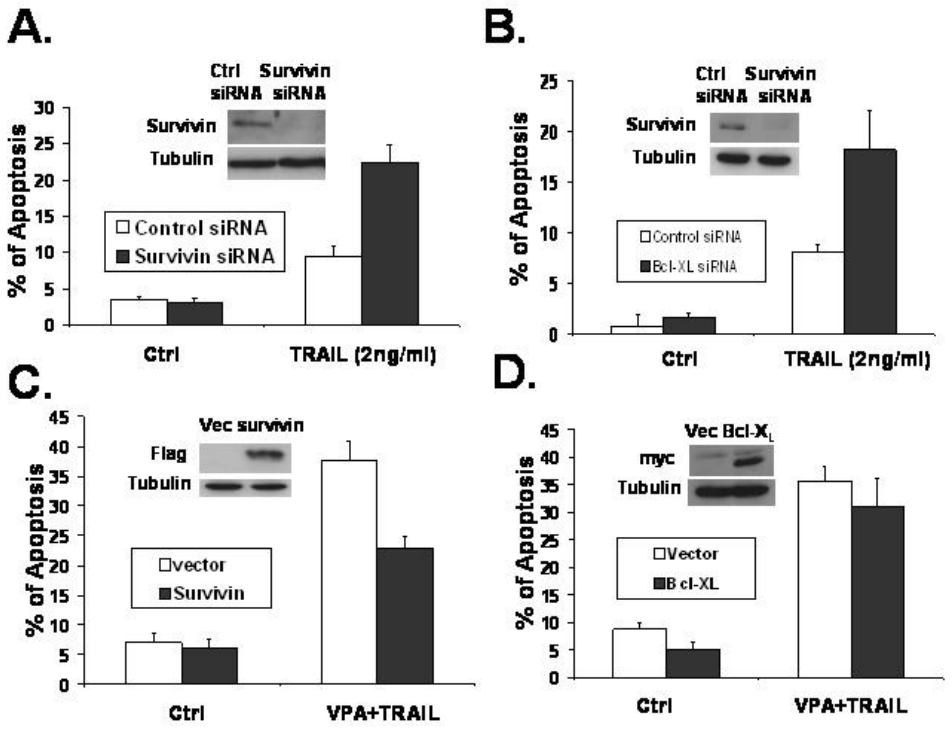

Figure 6. Survivin and Bcl-X_L regulate TRAIL-induced apoptosis. **A and B,** knocking-down of survivin and Bcl-X_L sensitize ZR-75-1 cells to TRAIL-induced apoptosis. ZR-75-1 cells were transfected with survivin (**A**) or Bcl-X_L (**B**) siRNA. 24 h after transfection, TRAIL was added for 24 h. Apoptosis was determined by DAPI staining. Protein expression was analyzed by western blotting. **C and D,** effects of overexpression of surviving and Bcl-X_L on apoptosis induced by combined treatment of VPA plus TRAIL. ZR-75-1 cells were transfected with plasmids expressing Flag-survivin or Myc-Bcl-X_L fusion proteins. After 24 h, cells were treated with 2mM VPA for 24 h and subsequently with TRAIL (2ng/ml) for 24 h. Apoptosis was determined by DAPI staining. Protein expression was analyzed by western blotting with anti-Flag and anti-myc antibodies.

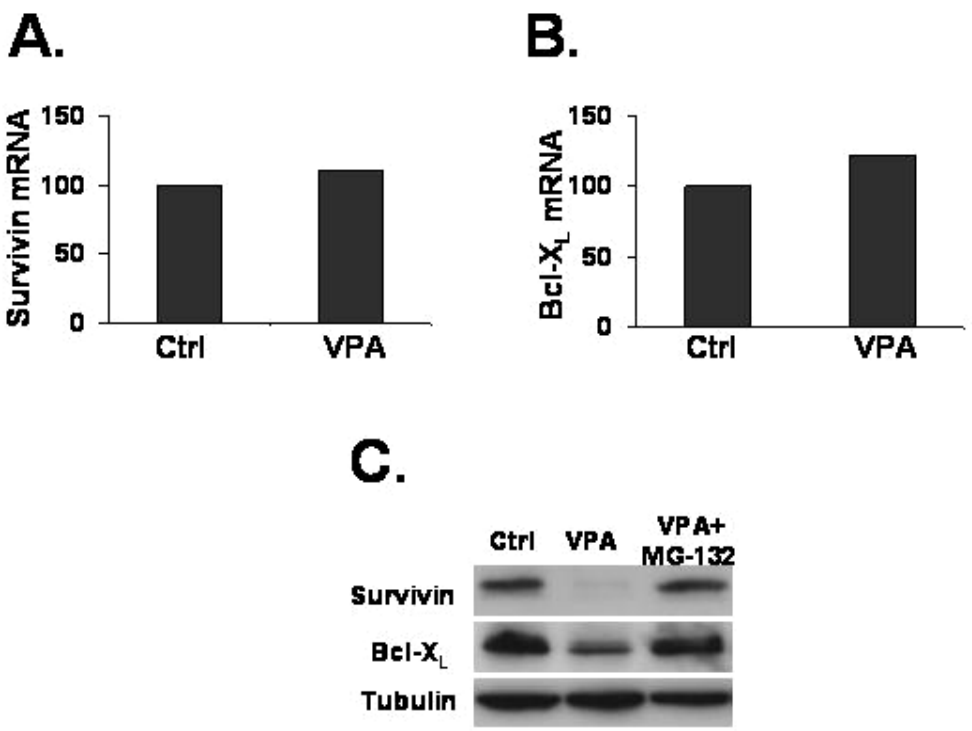

Figure 7. VPA down-regulates survivin and Bcl-X_L by increased protein degradation. **A and B,** ZR-75-1 cells were treated with 2mM VPA for 24 hours. The mRNA levels of survivin and Bcl-X_L were determined by real-time PCR. **C,** ZR-75-1 cells were treated with 2mM VPA for 48 h or with 2mM VPA for 40h and then followed by addition of proteasome inhibitor MG-132 (5μM) for 8h. Protein expression was analyzed by western blotting.